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| (54) Title: METHOD FOR CLONING SECRETED PROTEINS | | |
| <p>(57) Abstract</p> <p>The invention provides a method for isolating nucleic acids encoding proteins comprising a signal peptide, e.g. a secreted protein. The method of the invention comprises isolating an RNA molecule associated with an endoplasmic reticulum membrane preparation under conditions wherein the RNA is at least partially translated. The invention also provides a library of nucleic acids encoding proteins comprising a signal peptide, individual nucleic acids isolated according to the method of invention, peptides encoded thereby, pharmaceutical compositions comprising such and kits for performing the method of the invention.</p> | | |

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METHOD FOR CLONING SECRETED PROTEINS

5 Background of the Invention

Many biologically important molecules, in particular for use in therapy, are secreted proteins. For example, growth factors, interferons, erythropoietin, and insulin have been used successfully for treating various conditions and diseases.

10 Secreted proteins are characterized by the presence of a hydrophobic signal peptide at the amino terminus of the protein. The hydrophobic signal sequence is typically from about 16 to about 30 amino acids long and contains one or more positively charged amino acid residues near its N-terminus, followed by a continuous stretch of 6-12 hydrophobic residues. Signal peptides from various secreted proteins have otherwise no sequence homology. The presence of a hydrophobic signal peptide at the amino terminus of a protein mediates its association with the rough
15 endoplasmic reticulum (ER), which in turn mediates its secretion from the cell.

The mechanism by which peptides or proteins having a signal peptide associate with the endoplasmic reticulum and are secreted is as follows. Protein synthesis begins on free ribosomes. When the elongating peptide is about 70 amino acids long, the signal peptide is recognized by a particle, termed a "signal recognition particle" or "SRP", which in turn is capable of interacting with
20 a receptor, termed "SRP receptor", located on the ER. Thus, growing peptides having a signal peptide are targeted to the ER, where peptide synthesis continues on the rough ER. At some point during the protein synthesis or after the protein synthesis is completed, the protein is translocated across the ER membrane into the ER lumen, where the signal peptide is cleaved off. There the protein can be posttranslationally modified, e.g., glycosylated. Whether posttranslationally modified
25 or not, the protein can then be directed to the appropriate cellular compartment, e.g., secreted outside the cell.

Several systems have recently been developed to isolate nucleic acids encoding secreted proteins. One system which is used frequently and of which several variations exist is a system termed "Sequence Signal Trap". One such system (such as the Genetic Institute's DiscoverEase™
30 program) is yeast based and uses the yeast invertase gene, which cleaves the disaccharide sucrose

into monosaccharides glucose and fructose. According to the system, a library of cDNAs is cloned upstream of the gene encoding invertase and yeast cells are selected on sucrose. Since yeast cells cannot ingest sucrose, but can ingest fructose and glucose, only yeasts secreting invertase are able to grow on sucrose. Thus, only yeasts which contain a cDNA containing a signal sequence properly fused to the invertase gene will permit invertase to be secreted and will survive on sucrose.

5 However, such systems have several drawbacks. For example, the sequence signal trap system requires that the sequence signal be fused properly, e.g., in frame, to the gene encoding the invertase. Even where the signal sequence is in frame, this may produce a fusion protein, which may be instable. Thus, only a fraction of the cDNAs containing a signal sequence will be fused properly to the invertase gene to permit secretion of the invertase gene. Furthermore, this system
10 requires that the protein containing the signal sequence be secreted. However, it is known that numerous proteins containing signal peptides are trapped in the endoplasmic reticulum. Accordingly, the requirement that the fusion protein containing the signal peptide be actually secreted further reduces the efficiency of cloning secreted proteins.

15 Thus, it is highly desirable to have a system for isolating nucleic acids encoding secreted proteins which is more efficient and reliable than the existing sequence signal trap systems.

Summary of the Invention

20 The invention pertains to a method for isolation of nucleic acids encoding proteins comprising a signal peptide, e.g., secreted proteins. In a preferred embodiment, the invention comprises isolating an RNA molecule associated with microsomes. An even more preferred method is outlined in Figure 1. The method of the invention provides several advantages over known methods for cloning genes encoding secreted proteins. In particular, the method of the invention does not involve a fusion protein. Rather, the method selects natural secreted proteins. Furthermore, the method of the invention does not require the protein to be secreted.

25 The invention is based at least in part on the observation that nucleic acid molecules encoding secreted proteins can be cloned from RNA that is isolated from microsomes. As shown herein, an RNA molecule encoding a protein having a signal peptide can be isolated from a population of RNA molecules by *in vitro* translation of a population of RNA molecules, contacting the RNA molecules being translated with microsomes, purification of the microsomes from the free
30 ribosomes, and isolation of RNA from the purified microsomes.

Accordingly, the invention provides a method for isolating a nucleic acid encoding a protein having a signal peptide, such as a secreted protein. The method comprises isolating an RNA molecule associated with microsomes under conditions wherein the RNA is at least partially translated. In a preferred embodiment, the method further comprises synthesizing a single stranded

DNA molecule from the RNA molecule. The single stranded DNA molecule can further be converted into a double stranded DNA molecule. The RNA, single stranded or double stranded DNA molecule can be inserted in a vector, such as an expression vector. In one embodiment, the method further comprises producing a protein from the expression vector. The RNA, DNA, or protein can be used for preparing a pharmaceutical composition comprising a pharmaceutically acceptable carrier.

In one embodiment of the invention, the microsomes and the RNA are from the same cell, such as a mammalian cells, e.g., a human cell. In another embodiment, the microsomes and the RNA are heterologous.

In one instance, the RNA which is at least partially translated is an RNA which is translated in an *in vitro* translation system, such as a reticulocyte lysate. The RNA can be isolated from a cell or tissue sample, e.g., a human cell or tissue. However, the RNA can also be transcribed *in vivo*, i.e., the RNA that is at least partially translated is obtained from a cell.

In a preferred embodiment of the invention, RNA, an *in vitro* translation system and microsomes are contacted, to thereby produce RNA which is partially translated and wherein RNA encoding a protein comprising a signal peptide is associated with the microsomes. In an even preferred embodiment, the method further comprises isolating the microsomes and the RNA associated therewith, such as by sucrose density gradient centrifugation.

The invention also provides a method for enriching a population of RNA molecules in RNA molecules encoding a protein comprising a signal peptide, comprising (i) contacting the population of RNA molecules with a translation system, to thereby produce at least partially translated RNA molecules; (ii) contacting the at least partially translated RNA molecules with microsomes under conditions in which the partially translated RNA molecules comprising a signal peptide associate with the microsomes; (iii) isolating the microsomes and the RNA molecules associated with the microsomes; and (iv) isolating the RNA molecules from the microsomes, to thereby produce a population of RNA molecules enriched in RNA molecules encoding a protein comprising a signal peptide. The enrichment of the RNA population in RNA molecules containing a signal sequence can be of a factor of about 10, preferably at least about 100, at least about 10^3 , at least about 10^4 , at least about 10^5 , at least about 10^6 , at least about 10^7 or at least about 10^8 .

Also within the scope of the invention are libraries of nucleic acids encoding proteins comprising a signal peptide, prepared according to a method of the invention. The library can be a cDNA library or an expression library.

The invention further provides nucleic acids, such as nucleic acids encoding secreted proteins, isolated according to the method of the invention and peptides encoded thereby. Nucleic

acids and peptides of the invention can be prepared as a pharmaceutical preparation comprising a pharmaceutically acceptable carrier.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

5 **Brief Description of the Drawings**

Figure 1 outlines the steps of a preferred method of the invention for isolating RNA molecules encoding proteins containing a signal peptide.

Figure 2 is a photograph of an agarose gel showing RT-PCR amplified FRL2 and ODC RNA isolated from the top, middle and bottom of a sucrose gradient loaded with a translation
10 reaction with ("Plus") or without ("Minus") microsomes.

Figure 3 shows photographs from agarose gels containing RT-PCR amplified RNA encoding secreted proteins isolated from the top ("T"), or bottom ("B") of a sucrose gradient loaded with a translation reaction containing microsomes and RNA generated from a Stage 11-15 *Xenopus* library.

15 *Figure 4* shows photographs from agarose gels containing RT-PCR amplified RNA encoding cytosolic proteins isolated from the top ("T"), or bottom ("B") of a sucrose gradient loaded with a translation reaction containing microsomes and RNA generated from a Stage 11-15 *Xenopus* library.

20 **Detailed Description of the Invention**

 The endoplasmic reticulum (ER) of eukaryotic cells provides the cells with a mechanism for separating newly synthesized molecules that belong to the cytoplasm from those that do not. Lipids, proteins and complex carbohydrates destined for transportation to the Golgi apparatus, to the plasma membrane, to lysosomes, or to the cell exterior are all synthesized in association with
25 the ER. Association of proteins with rough ER is mediated through the presence of a hydrophobic signal peptide at the amino terminus of the protein.

 The ER has two functionally and structurally distinct regions: the rough endoplasmic reticulum, which is covered with ribosomes on the cytoplasmic side of the membrane and the smooth endoplasmic reticulum, which lacks ribosomes. The rough endoplasmic ribosome is
30 involved in the synthesis of secretory proteins, integral, ER, Golgi, and plasma-membrane proteins, glycoproteins and lysosome proteins. Though all nucleated cells, except sperm cells, have ER, the amount of rough ER varies from one cell type to another, depending of the function of the cell. For example, a cell specialized in protein secretion, such as a pancreatic acinar cell and antibody secreting plasma cell, or a cell undergoing extensive membrane synthesis, e.g., an immature egg or

a retinal rod cell, are particularly rich in rough ER. The smooth ER is not involved in protein synthesis.

Upon disruption of a tissue or cells by homogenization, the ER is fragmented into many smaller (about 100 nm diameter) closed vesicles called "microsomes", which are relatively easy to purify. Microsomes derived from the rough ER, are covered with ribosomes on the outside of the
5 microsomes and are termed "rough microsomes". Such a tissue or cell homogenate also contains many vesicles of a size similar to the rough microsomes, but which do not contain ribosomes on their surface. Such smooth microsomes are derived in part from the smooth portions of the ER and in part from vesiculated fragments of plasma membranes, Golgi apparatus, and mitochondria. Rough microsomes can be separated from smooth microsomes, e.g., by sucrose gradient
10 centrifugation. In fact, smooth microsomes have a low density and stop sedimenting and float at a low sucrose concentration, whereas rough microsomes have a high density and stop sedimenting and float at high sucrose concentration.

The present invention provides a method and reagents for isolating a nucleic acid encoding a protein having a signal peptide, by isolating an RNA molecule from microsomes or other ER
15 preparation. In a preferred embodiment, the protein having a signal peptide is a secreted protein. The protein can also be an integral, ER, Golgi, plasma-membrane protein, a glycoprotein, or a lysosome protein. In another embodiment, a population of RNA molecules is isolated from microsomes and used to prepare a library of nucleic acids encoding proteins having a signal peptide. In a preferred embodiment of the invention, the library is a cDNA library. In yet another
20 embodiment, the invention further comprises isolating individual members of the library, e.g., cDNAs encoding secreted proteins.

In an exemplary embodiment of the invention, the RNA molecule or the population of RNA molecules isolated from microsomes and the microsomes are heterologous, i.e., originate from different sources. For example, a population of RNA molecules being translated can first be
25 contacted with heterologous microsomes and one or more RNA molecule associating with the microsomes can be isolated. In one embodiment, the RNA is first incubated with an *in vitro* transcription system and then microsomes are added to the *in vitro* transcription reaction. Alternatively, the RNA is added to a mixture containing an *in vitro* translation system and microsomes.

30

Definitions

For convenience, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided below.

The phrase "at least partially translated RNA molecule" refers to an RNA molecule which is associated with at least one ribosome and which has served as a template for the synthesis of a peptide of sufficient length to be capable of associating with the endoplasmic reticulum (ER), e.g., by interaction with SRP receptors or by being inserted into the ER membrane. Accordingly, a partially translated RNA molecule will have been translated into a polypeptide of at least about 50 amino acids, preferably at least about 60 amino acids, at least about 70 amino acids, at least about 80 amino acids, at least about 90 amino acids or at least about 100 amino acids. In fact, the N-terminal signal sequence emerges from the ribosome only when the polypeptide is about 70 amino acids long, since about 30 amino acids remain buried in the ribosome (James Darnell, Harvey Lodish, and David Baltimore, *Molecular Cell Biology* (1990) Scientific American Books). In a preferred embodiment, the at least partially translated RNA molecule is not completely translated, since in this case the ribosome/RNA/polypeptide complex dissociates from the ER membrane.

The phrase "converting a single stranded DNA molecule into a double stranded DNA molecule" refers to any molecular process whereby a single stranded DNA molecule is rendered double stranded. Such a molecular process can be, e.g., primer extension from a primer hybridizing to the single stranded DNA molecule.

The term "*in vitro* translation system", which is used herein interchangeably with the term "cell-free translation system" refers to a translation system which is a cell-free extract containing at least the minimum elements necessary for translation of an RNA molecule into a protein. An *in vitro* translation system typically comprises at least ribosomes, tRNAs, initiator methionyl-tRNA^{Met}, proteins or complexes involved in translation, e.g., eIF₂, eIF₃, the cap-binding (CB) complex, comprising the cap-binding protein (CBP) and eukaryotic initiation factor 4F (eIF_{4F}).

The term "isolated" as used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs or RNAs, respectively, that are present in the natural source of the macromolecule. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides.

The term "heterologous" when referring to ER preparations, such as microsomes, and RNA means that the ER preparations and the RNA are not from the same cell, i.e., that the ER preparations are from one source of cells and the RNA is RNA isolated from a second source or is synthesized *in vitro*. The ER preparations and the RNA can be heterologous with respect to each

other if they originate from the same cell or tissue, so long as the RNA and the ER preparations have been separated (enriched separately from one another) prior to being contacted.

As used herein, the term "microsome" refers to a rough microsome, as opposed to a smooth microsome. Accordingly, a "microsome" contains SRP receptors which are capable of interacting with an SRP complex which itself interacts with a signal peptide.

5 As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, derivatives, variants and analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

10 The term "operably linked" is intended to mean that the promoter is associated with the nucleic acid in such a manner as to facilitate transcription of the nucleic acid from the promoter.

The terms "protein", "polypeptide" and "peptide" are used interchangeably herein when referring to the translation product of a nucleic acid (e.g. a gene product).

15 The term "RNA" refers to a nucleic acid in which the sugar is ribose, as opposed to deoxyribose in DNA. RNA is intended to include any nucleic acid which can be entrapped by ribosomes and translated into protein.

The term "recombinant protein" refers to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein.

20 The term "secreted protein" refers to a protein having at least a portion which is extracellular and includes proteins which are completely extracellular (i.e., not attached to a cell) and proteins which are cytoplasmic membrane proteins. The cell can be a prokaryotic cell, e.g., bacterial cell or the cell can be a eukaryotic cell.

25 The term "signal peptide" refers to a stretch of amino acids mediating the attachment of a protein to the ER, such that the protein having a signal peptide is secreted or inserted into a cell membrane.

The term "signal sequence" refers to a nucleotide sequence encoding a signal peptide.

The term "soluble protein" refers to a secreted protein which is completely extracellular and which are not embedded in a cell membrane.

30 As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. The term "transduction" is generally used herein when the transfection with a nucleic acid is by viral delivery of the nucleic acid.

"Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of a polypeptide or, in the case of anti-sense expression from the transferred gene, the expression of a naturally-occurring form of the recombinant protein is disrupted.

5 The term "translation system" refers to a cellular or cell-free translation system. The term "cellular translation system" refers to a translation system which is a permeabilized cell, as opposed to a "cell-free translation system", which refers to a cell extract or a reconstituted translation system. The term "reconstituted translation system" refers to a system consisting of purified translation factors, such as elongation factors.

10 As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to
15 herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression
20 vectors which serve equivalent functions and which become known in the art subsequently hereto.

Exemplary sources of, and methods of obtaining, RNA

In a preferred embodiment, a population of RNA molecules (RNA) is contacted with an *in vitro* translation system together, before, or after incubation with microsomes. The population of
25 RNA molecules can be from a cell or a tissue. In a preferred embodiment, the RNA is from a eukaryote. In an even more preferred embodiment, the RNA is from a vertebrate, e.g., a mammal. In a yet even more preferred embodiment, the RNA is from a human.

Preferred cells and tissues from which RNA can be extracted for use in the method of the invention include RNA from tissues which are known to secrete numerous proteins, e.g., exocrine
30 or endocrine tissues. Preferred tissues include glands, exocrine glands, e.g., pancreas, salivary glands, submandibular gland, and others. Other preferred tissues include endocrine tissues, e.g., thyroid.

RNA can be extracted from cells or tissues according to methods known in the art. In a preferred embodiment, RNA can be extracted from monolayers of mammalian cells grown in tissue

culture, cells in suspension or from mammalian tissue that can readily be dispersed in single cells. RNA can be extracted from such sources by, e.g., treating the cells with proteinase K in the presence of SDS. In another embodiment, RNA is extracted by organic solvents. In yet another embodiment, RNA is extracted by differential precipitation to separate high molecular weight RNA from other nucleic acids. RNA can also be extracted from a specific cellular compartment, e.g., nucleus or the cytoplasm. In such methods, the nucleus is either isolated for purification of RNA therefrom, or the nucleus is discarded for purification of cytoplasmic RNA. Further details regarding these and other RNA extraction protocols are set forth, e.g., in *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989).

For instance, RNA can be extracted by a method using guanidium thiocyanate and purification of the RNA on a cesium chloride gradient. Accordingly, tissue or cells are lysed in the presence of guanidium thiocyanate and the cell lysate is loaded on a cushion of cesium chloride (CsCl) and centrifuged at high speed, such that the RNA is recovered in the pellet and the DNA is left in the supernatant after the centrifugation. The RNA can then be recovered by ethanol precipitation. This method is set forth in details, e.g., in *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989).

In order to prevent RNA from being degraded by nucleases, e.g., by RNAases, that may be present, the extraction of RNA, and reactions involving RNA are performed in "RNAase free conditions". Various methods known in the art can be used to maintain RNAase free conditions. For example, during RNA extraction, potent denaturing agents, such as guanidium hydrochloride and guanidium thiocyanate can be used to denature and thereby inactivate nucleases. Reducing agents, e.g., β -mercaptoethanol, can also be used to inactivate ribonucleases. This combination of agents is particularly useful when isolating RNA from tissues rich in ribonucleases, e.g., pancreas (Chirgwin et al. (1979) *Biochemistry* 18:5294).

Other reagents that can be added to a solution containing RNA to prevent degradation of the RNA include RNAase inhibitors, also referred to herein as "protein inhibitor of RNAases", e.g., RNasin® which can be obtained, from Promega Corp. (Madison, Wis.) (e.g., Cat # N2514). Protein inhibitors of RNAases are preferably not included during extraction of RNA using potent denaturing agents (since these will also denature the protein inhibitor of RNAases). However, it is preferable to include such protein inhibitors of RNAases during RNA extraction using more gentle methods of cell lysis and RNAase inhibitors are preferably present at all stages during the subsequent purification of RNA.

Yet another reagent that can be added to a solution containing RNA to prevent degradation of the RNA include vanadyl-ribonucleoside complexes. The complexes formed between the

oxovanadium IV ion and any of the four ribonucleosides are transition-state analogs that bind to many RNAases and inhibit their activity almost completely. The four vanadyl-ribonucleoside complexes are preferably added to intact cells and preferably used at a concentration of 10 mM during all stages of RNA extraction and purification. Yet in another embodiment, macaloid is used to absorb RNAases.

5 Prior to contacting the RNA with a translation system and microsomes, the RNA can be partially purified. For example, the polyA⁺ RNA can be isolated from total RNA by, e.g., oligodT affinity chromatography, according to methods known in the art. Use of polyA⁺ RNA relative to total RNA may improve the translation efficiency in certain situations.

10 In another embodiment, RNA or polyA⁺ RNA having a specific molecular size can be isolated. Thus, if one desires to isolate an RNA molecule encoding a small secreted protein, RNA molecules of small sizes can be isolated and incubated with a translation system and microsomes. For example, RNA of sizes of about or less than 1 kb, about 2 kb, about 3 kb, about 4 kb, about 5 kb, about 6 kb, about 7 kb, about 8 kb, about 8 kb, about 9 kb or at least about 10 kb can be selected. RNA having a size between about 1 and 3 kb, between about 3 and 5 kb, or between
15 about 5 and 10 kb can also be isolated. Size fractionation of RNA can be performed, e.g., by electrophoresis through agarose gels. After the electrophoresis, the gels can be stained, e.g., with ethidium bromide, to visualize the RNA, and RNA having the desired size can be recovered from the gel. Ribosomal RNA of known size can be used as molecular weight standards. Gel electrophoresis is preferably carried out in the presence of an RNAase inhibitor, e.g.,
20 methylmercuric hydroxide.

Another method that can be used to isolate RNA of a specific size is by centrifugation through a sucrose gradient, e.g., containing methylmercuric hydroxide. According to this method, an RNA solution is loaded onto a sucrose gradient (10-30% weight/volume) containing about 10 mM methylmercuric hydroxide and centrifuged, at 34,000rpm for about 15 hours. A gradient of
25 RNA molecules, reflecting the size of the RNA molecules, will be formed in the tube after centrifugation. Fractions can then be collected and the RNA isolated, e.g., by ethanol precipitation.

In another embodiment, the source of RNA from which RNA molecules encoding proteins containing signal peptides are isolated, is obtained by *in vitro* transcription of template DNA. For example, the RNA can be synthesized *in vitro* from a DNA library, e.g., cDNA library.
30 Alternatively, the RNA can be synthesized from another source of DNA, e.g., genomic DNA. In a preferred embodiment of the invention, the DNA to be *in vitro* transcribed is operably linked to, and is located downstream of, an RNA polymerase promoter, e.g., a bacteriophage promoter, such as SP6, T3 or T7 promoter. In one embodiment, the DNA to be transcribed is cloned in a cDNA library at a site located downstream of a bacteriophage promoter and transcription is carried out

from the library itself, resulting in a library of RNA molecules. If the DNA to be transcribed is in a vector which does not contain a bacteriophage promoter, the cDNA inserts can first be excised from the vector and cloned in another vector from which the inserts can be *in vitro* transcribed.

Many vectors for *in vitro* transcription are available commercially. These may contain one or more of the promoters SP6, T3 and T7 and may additionally contain a polyA sequence at the 3' end of the polylinker in which the DNA of interest is inserted. A "polylinker" refers to a nucleotide sequence containing several restriction enzyme recognition sites. Examples of vectors include the series of SP6 vectors, e.g., SP64 (Krieg and Melton, *infra*), BlueScript, and pCS2+. Vectors that can be used for *in vitro* transcription are also described, e.g., in U.S. Patent No. 4,766,072.

An *in vitro* transcription reaction can be carried out according to methods well known in the art. Kits for performing *in vitro* transcription kits are also commercially available from several manufacturers. In an illustrative embodiment, an *in vitro* transcription reaction is carried out as follows. A vector containing an RNA Polymerase promoter and an insert of interest is preferably first linearized downstream of the insert, by e.g., restriction digest with an appropriate restriction enzyme. The linearized DNA is then incubated for about 1 hour at 37 or 40°C (depending on the RNA polymerase) in the presence of ribonucleotides, an RNAase inhibitor, an RNA polymerase recognizing the promoter that is operably linked upstream of the insert to be transcribed, and an appropriate buffer containing Tris.Cl, MgCl₂, spermidine and NaCl. Following the transcription reaction, RNAase free DNase can be added to remove the DNA template and the RNA can be purified by, e.g., a phenol-chlorophorm extraction. Usually about 5-10 µg of RNA can be obtained per microgram of template DNA. Further details regarding this protocol are set forth, e.g., in Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989).

In another embodiment, the RNA is "capped" prior to contacting it with an *in vitro* translation system. In certain situations, efficient translation of eukaryotic RNA requires that the 5' end of an RNA molecule is "capped", i.e., that the 5' nucleotide at the 5' end of the RNA has a 5'-5' linkage with a 7-methylguanylate ("7-methyl G") residue. The presence of a 7-methyl G on an RNA molecule in a 5'-5' linkage is referred to as a "cap". It has been proposed that recognition of the translational start site in mRNA by the eukaryotic ribosomes involves recognition of the cap, followed by binding to specific sequences surrounding the initiation codon on the RNA. Accordingly, it is possible that in certain embodiments of the invention, capping of the RNA extracted from a cell or tissue or synthesized *in vitro* prior to contacting the RNA with an *in vitro* translation system improves the translation efficiency of the RNA and thus the amount of RNA eventually recovered from the microsomes. Thus, in one embodiment, the RNA is contacted with methyl-7 (5')PPP(5')guanylate (available, e.g., from Boehringer Mannheim Biochemicals) in the

presence of an *in vitro* transcription reaction mixture, to obtain capped RNA. In the case of *in vitro* transcribed RNA, capping is preferably carried out during *in vitro* transcription, but can also be carried out during *in vitro* translation by, e.g., addition of a cap analog (GpppG or a methylated derivative thereof). Cap analogs and protocols pertaining to their use are commercially available, e.g. in *in vitro* transcription and/or translation kits.

- 5 The RNA for use in the invention preferably comprises most of the 5' end, at least enough of the 5' end of the RNA to contain a sufficiently long stretch of the signal sequence to encode a signal peptide that is sufficiently long to be capable of interacting with an SRP.

Exemplary translation systems

- 10 In order for RNA encoding proteins having a signal peptide to become attached to the ER or to microsomes, the RNA must be in the process of being translated. In fact, RNA sequence encoding the signal peptide must be essentially fully translated to allow attachment of the signal peptide to the SRP complex, which can then interact with an SRP receptor located on the outer surface of the rough ER or rough microsomes. Translation systems may be cellular or cell-free, and
15 may be prokaryotic or eukaryotic cellular translation systems. Cellular translation systems, as used herein, refer to permeabilized cells. Cell-free translation systems, also referred to as "*in vitro* translation systems", refer to cell extracts and reconstituted systems.

- In a preferred embodiment, the translation system is an *in vitro* translation system. A variety of *in vitro* translation systems are well known in the art and include commercially available
20 kits. Examples of *in vitro* translation systems include eukaryotic lysates, such as rabbit reticulocyte lysates, rabbit oocyte lysates, human cell lysates, insect cell lysates and wheat germ extracts. Lysates are commercially available from manufacturers such as Promega Corp., Madison, Wis.; Stratagene, La Jolla, Calif.; Amersham, Arlington Heights, Ill.; and GIBCO/BRL, Grand Island, N.Y. *In vitro* translation systems typically comprise macromolecules, such as enzymes, translation,
25 initiation and elongation factors, chemical reagents, and ribosomes.

 Mixtures of purified translation factors, as well as combinations of lysates or lysates supplemented with purified translation factors such as initiation factor-1 (IF-1), IF-2, IF-3 (alpha or beta), elongation factor T (EF-Tu) or termination factors can also be used for translation of RNA.

- 30 Translations in cell-free systems generally require incubation of the ingredients for a period of time sufficient to allow translation of mRNA into a polypeptide of sufficient length to enable the polypeptide to interact with a SRP complex. Incubation times may range from about 5 minutes to many hours, but is preferably between about thirty minutes to about five hours and more preferably between about one to about three hours. Incubation times vary significantly with the volume of the

translation mix and the temperature of the incubation. Incubation temperatures can be between about 4°C to about 60°C, and are preferably between about 15°C to about 50°C, and more preferably between about 25°C to about 45°C and even more preferably at about 25°C or about 37°C. Translation mixes will typically comprise buffers such as Tris-HCl, Hepes or another suitable buffering agent to maintain the pH of the solution between about 6 to 8, and preferably at about 7. Other reagents which may be included in the translation system include dithiothreitol (DTT) or 2-mercaptoethanol as reducing agents, RNasin to inhibit RNA breakdown, and nucleoside triphosphates or creatine phosphate and creatine kinase to provide chemical energy for the translation process. Preferred translation systems and translation conditions are further described herein.

Translation may also be performed in a continuous manner wherein reagents are flowed into the system and nascent proteins removed or left to accumulate using a continuous flow system (A. S. Spirin et al., Science 242:1162-64, 1988). This process may be desirable for large scale production of nascent proteins.

In an illustrative embodiment, the *in vitro* translation system is a reticulocyte lysate, which is available commercially or can be prepared, e.g., according to methods known in the art and briefly outlined below. Commercially available reticulocyte lysates are available from, for example, New England Nuclear and Promega Corp. (Madison, Wis.) (Cat. # L4960, L4970, and L4980). In another embodiment, the *in vitro* translation system is a wheat germ translation system. Such a system is also available commercially or can be prepared according to methods known in the art. Commercially available wheat germ extracts can be obtained, e.g., from Promega Corp. (Madison, Wis.) (e.g., Cat # L4370). In yet another preferred embodiment, the *in vitro* translation system is a mixture of a reticulocyte lysate and a wheat germ extract. Such a mixture can be obtained commercially, e.g., from Promega Corp. (Madison, Wis.) (e.g., Cat.# L4340). Other *in vitro* translation systems that can be used according to the method of the invention include *Escherichia coli* extracts, insect cell extracts and frog oocytes extracts.

To further illustrate, *in vitro* translation systems can be prepared by several methods. For example, a rabbit reticulocyte lysate can be prepared as follows. Rabbits are rendered anemic by inoculation with acetylphenylhydrazine. About 7 days later, the rabbits are bled and the blood is collected and mixed with an ice cold salt solution containing NaCl, Mg acetate, KCl, and heparin. The blood is then filtered through a cheesecloth, centrifuged, and the buffy coat of white cells is removed. The pellet consisting of erythrocytes and reticulocytes is then washed with the salt solution, prior to being lysed by the addition of an equal volume of cold water. Endogenous RNAs are degraded by treating the lysate with micrococcal nuclease and calcium ions (necessary for the activity of the enzyme) and the reaction stopped by the addition of EGTA, which chelates the CaCl_2 .

and thereby inactivate the nuclease. Hemin is usually added to the lysate (at a concentration of about 20-80 μ M), since it is a powerful suppressor of an inhibitor of the initiation factor eIF-2. The lysates can further be optimized by the addition of an energy generating system, consisting of phosphocreatine kinase and phosphocreatine. The lysates can then be aliquoted and stored at -70°C or in liquid nitrogen. Further details regarding this protocol are set forth, e.g., in Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989).

A wheat germ extract can be prepared, e.g., by the method described by Roberts, B. E. and Paterson, B. M. (1973), Proc. Natl. Acad. Sci. USA Vol. 70, No. 8, pp. 2330-2334), which optionally can be modified according to Anderson, C. W., et al. (1983, Meth. Enzymol. Vol. 101, p. 635). This protocol can further be modified according to the manufacturing protocol L418, Promega Corp. Madison, Wis. Generally, wheat germ extract is prepared by grinding wheat germ in an extraction buffer, followed by centrifugation to remove cell debris. The supernatant is then separated by chromatography from endogenous amino acids and plant pigments that are inhibitory to translation. The extract is also treated with micrococcal nuclease to destroy endogenous mRNA, to reduce background translation to a minimum. The extract contains the cellular components necessary for protein synthesis, such as tRNA, rRNA and initiation, elongation, and termination factors. The extract can further be optimized by the addition of an energy generating system consisting of phosphocreatine kinase and phosphocreatine, and magnesium acetate is added at a level recommended for the translation of most mRNA species. The final magnesium concentration for standard wheat germ extract, is typically in the range of about 6.0 to 7.5 mM.

A wheat germ cell *in vitro* translation system can also be prepared as follows. Wheat germ embryo extracts are floated to enrich for embryos using a mixture of cyclohexane and carbon tetrachloride (1:6), followed by drying overnight (about 14 hours). Floated wheat germ embryos (5 g) are then grounded in a mortar with 5 grams of powdered glass to obtain a fine powder. Extraction medium (Buffer I: 10 mM trisacetate buffer, pH 7.6, 1 mM magnesium acetate, 90 mM potassium acetate, and 1 mM DTT) is added to small portions until a smooth paste is obtained. The homogenate containing disrupted embryos and 25 ml of extraction medium is centrifuged twice at 23,000 x g. The extract is applied to a Sephadex G-25 fine column and eluted in Buffer II (10 mM trisacetate buffer, pH 7.6, 3 mM magnesium acetate, 50 mM potassium acetate, and 1 mM DTT). A bright yellow band migrating in void volume is collected (S-23) in one ml fractions and frozen in liquid nitrogen for conservation prior to use in *in vitro* translation reactions.

An *in vitro* translation reaction using a reticulocyte lysate can be carried out as follows. Ten μ l of a reticulocyte lysate, e.g., prepared as set forth above or a commercial lysate, is mixed with spermidine, creatine phosphate, amino acids, HEPES buffer (pH 7.4), KCl, magnesium acetate and

RNA to be translated and incubated for an appropriate time, e.g., one hour, at 30°C. The optimum amount of magnesium acetate for obtaining efficient translation will vary from one reticulocyte lysate to another, and can be determined using a standard preparation of RNA and concentration of magnesium acetate varying from 0-1 mM. The optimal concentration of KCl can also be different depending on the specific reaction. For example, 70 mM KCl is usually optimal for translation of capped RNAs and 40 mM is usually optimal for translation of uncapped RNAs. To follow the translation process, one can add one or more amino acids as radioactive amino acids, e.g., ³⁵S methionine (further described below). In this case, the translation process can be monitored by precipitating the proteins in the lysate, e.g., TCA precipitating and counting the amount of radioactivity present in the precipitate. The translation products can also be analyzed by immunoprecipitation and/or SDS-polyacrylamide gel electrophoresis. Further details regarding this protocol are set forth, e.g., in Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989).

Translations in wheat germ extracts can be performed as described, e.g., in Erickson and Blobel (1983) Methods Enzymol. 96:38. This protocol can also be modified, e.g., by adjusting the final ion concentrations at 2.6 mM magnesium and 140 mM potassium and the pH at 7.5 (as described in U.S. Pat. No. 4,983,521). In a preferred embodiment, reaction mixtures are incubated at 24°C for 60 minutes. Translations in wheat germ extracts can also be performed according to the protocol set forth in U.S. Patent No. 5,492,817.

Translation reactions can be optimized by the addition of ions and/or other reagents. For example, magnesium is known to be important for optimizing translation, as it enhances the stability of assembled ribosomes and functions in their binding together during translation. Magnesium also appears to play a role in facilitating polymerase binding. Potassium is important as well for optimizing translation, but unlike the case for magnesium, for coupled transcription and translation the concentration of potassium ions does not need to be altered beyond standard translation preparation levels.

Potassium and magnesium are present in the standard rabbit lysate and are provided in part by the lysate itself, and in part by the addition of exogenous potassium and magnesium. As the magnesium concentration should be adjusted to within a rather narrow optimal range, it is preferred that the lysate magnesium levels be measured directly through the use of a magnesium assay, prior to the addition of extra magnesium, so that the amount of magnesium in a reaction can be standardized from one batch of lysate to the next. The Lancer "Magnesium Rapid Stat Diagnostic Kit" (Oxford Lab Ware Division, Sherwood Medical Co., St. Louis, Mo.), is one such assay which can accurately measure the magnesium levels in biological fluid. Once the magnesium ion concentration for a given batch of lysate is known then additional magnesium, for instance in the

form of a concentrated magnesium salt solution, can be added in a known manner to bring the magnesium concentration of the lysate to within the optimal range, or, in the case of a modified lysate preparation to be used as one-half of a reaction mixture, to within twice the optimal range. Magnesium concentration can be adjusted by using, e.g., a concentrated solution of magnesium chloride or magnesium acetate.

5 One common addition to translation reactions is an amount of a polyamine sufficient to stimulate the efficiency of chain elongation. For example, spermidine can be added to a reticulocyte lysate translation reaction to a final concentration of about 0.2 mM. Spermidine is also preferably added to wheat germ extracts, preferably at a concentration of about 0.9 mM. Since the presence of polyamines are known to lower the effective magnesium concentration for translation reactions,
10 the presence of spermidine in translation reactions should be taken into consideration when determining the appropriate concentration of magnesium to use.

In another embodiment, dithiothreitol (DTT) is added to the translation mixture. DTT is preferably added to a final concentration of about 1.45 mM in reticulocyte lysates and at 5.1 mM in wheat germ extracts.

15 Translation systems can also be supplemented with certain factors, e.g., tRNA molecules. Such molecules are commercially available, e.g., from a number of sources and can be prepared using well-known methods from sources including *Escherichia coli*, yeast, calf liver and wheat germ cells (Sigma Chemical; St. Louis, Mo.; Promega Corp.; Madison, Wis.; Boehringer Mannheim Biochemicals; Indianapolis, Ind.). Their isolation and purification mainly involves cell-lysis, phenol
20 extraction followed by chromatography on DEAE-cellulose. Amino-acid specific tRNA, for example tRNA^{fMet}, can be isolated by expression from cloned genes and overexpressed in host cells and separated from total tRNA by techniques such as preparative polyacrylamide gel electrophoresis followed by band excision and elution in high yield and purity (Seong and RajBhandary, Proc. Natl. Acad. Sci. USA 84:334-338, 1987).

25 Translation efficiency can also be improved, e.g., by adding RNAase inhibitors, e.g., RNasin® or heparin to the translation reaction. RNasin® can be obtained, e.g., from Promega Corp. (Madison, Wis.) (e.g., Cat # N2514). In a preferred embodiment about 40 units of RNasin® are added to a 50 µl reaction. Although the addition of RNAase inhibitors in reticulocyte lysates is not crucial, translation in a wheat germ extract is reduced if no RNAase inhibitor is added.

30 The translation reaction is preferably carried out from about 30 minutes to about 120 minutes prior to the addition of microsomes or in the presence of microsomes, such that a sufficient amount of the signal peptide has been translated to allow its binding to the SRP on the microsomes. The optimum time of translation can be determined, e.g., by labeling the RNA, e.g., by *in vitro* transcribing RNA in the presence of a labeled, e.g., radioactively labeled ribonucleotide, contacting

the labeled RNA with an *in vitro* translation system, and measuring the amount of label associated with the microsomes over time. The optimal time is the time at which the maximum amount of label, thus RNA, is associated with the microsomes.

5 In a preferred embodiment, the translation process, including the movement of the ribosomes on the RNA molecules, is inhibited at an appropriate time by the addition of an inhibitor or translation, e.g., cycloheximide. Accordingly, in one embodiment, cycloheximide is added at a final concentration of 1 $\mu\text{g/ml}$ to the translation reaction, which contains or does not contain microsomes. Mg^{2+} , e.g., MgCl_2 , e.g., at a concentration of 5 mM can also be added to maintain the mRNA-80s ribosome-nascent polypeptide complexes (polysomes).

10 Translation of mRNA in an *in vitro* system can be followed, e.g., by including a labeled amino acid in the translation reaction, and using an amino acid mixture depleted in this specific amino acid. A preferred labeled amino acid is a radioactively labeled amino acid, e.g., ^{35}S methionine. Alternatively, a labeled non-radioactive amino acid can be incorporated into the nascent polypeptide. For example, the translation reaction can comprise a misaminoacylated tRNA, as described in U.S. Patent No. 5,643,722. Accordingly, a non-radioactive marker, is
15 misaminoacylated to a tRNA molecule and this tRNA is added to the translation system. The system is incubated to incorporate the marker into the nascent polypeptide and polypeptides containing the marker can be detected using a detection method appropriate for the specific marker.

Useful markers are native amino acids coupled with a detectable label, detectable non-native amino acids, detectable amino acid analogs and detectable amino acid derivatives. Labels and other
20 detectable moieties may be ferromagnetic, paramagnetic, diamagnetic, luminescent, electrochemiluminescent, fluorescent, phosphorescent or chromatic. Fluorescent moieties which are useful as markers include dansyl fluorophores, coumarins and coumarin derivatives, fluorescent acridinium moieties and benzopyrene based fluorophores. Preferably, the fluorescent marker has a high quantum yield of fluorescence at a wavelength different from native amino acids. Upon
25 excitation at a preselected wavelength, the marker is detectable at low concentrations either visually or using conventional fluorescence detection methods. Electrochemiluminescent markers such as ruthenium chelates and its derivatives or nitroxide amino acids and their derivatives are preferred when extreme sensitivity is desired (J. DiCesare et al., *BioTechniques* 15:152-59, 1993). These markers are detectable at the femtomolar ranges and below. Biotin, streptavidin and derivatives
30 thereof can also be used to label amino acids. These and other markers for labeling amino acids for detection of nascent polypeptides are disclosed in U.S. Patent No. 5,643,722.

In another embodiment of the invention, the *in vitro* transcription and translation reactions are performed simultaneously. This can be done, e.g., by using commercially available systems, such as the Coupled Transcription/Translation System of Promega Corp. (Madison, Wis.) (Cat #

L4600, 4610, and 4950). Coupled transcription and translation systems using RNA polymerases and eukaryotic lysates are further described in U.S. Patent No. 5,324,637.

Cell-based translation systems, e.g., permeabilized cells, can be prepared as follows. Cells can be permeabilized by incubation for a short period of time in a solution containing low concentrations of detergents in a hypotonic media. Useful detergents include Nonidet-P 40 (NP40), Triton X-100 (TX-100) or deoxycholate at concentrations of about 0.01 nM to 1.0 mM, preferably between about 0.1 μ M to about 0.01 mM, and more preferably about 1 μ M. Such systems can be formed from intact cells in culture such as bacterial cells, primary cells, immortalized cell lines, human cells or mixed cell populations.

10 Exemplary ER preparations

In preferred embodiments, the ER preparations used to generate the subject reaction systems are microsomal preparations. Microsomes can be added to the *in vitro* translation system at the same time as the RNA, such that the RNA becomes attached to the microsomes as soon as the length of the growing peptidic chain is sufficient for allowing attachment of the signal peptide to the SRP. Alternatively, translation can be carried out for a specific amount of time and the microsomes can then be added to the translation reaction. Optionally, inhibitors of translation can be added at an appropriate time to the reaction. In one embodiment, the RNA is contacted with an *in vitro* translation system for about 30-120 minutes prior to the addition of microsomes. As set forth above, the optimum amount of time for incubation of the RNA with an *in vitro* translation system can be determined, e.g., by performing small scale assays in which the RNA is labeled, translated for various amounts of time, contacted with microsomes, and the amount of labeled RNA attached to the microsomes is determined.

Microsomes are commercially available. For example, canine pancreatic microsomal membranes are available from Promega Corp. (Madison, Wis.) (Cat. # Y4041). Microsomes for use in the invention, can also be prepared according to methods known in the art, e.g., as described in Walter and Blobel (1983) Methods Enzymol. 96:682), or a modification thereof, as described in U.S. Patent No. 4,983,521. Briefly, a cell or tissue can be homogenized, subjected to low speed centrifugation to pellet nuclei, mitochondria, and cell debris, leaving the microsomes (rough and smooth) in suspension. The supernatant can then be subjected to a sucrose density gradient, which after centrifugation to equilibrium results in the separation of the rough and smooth microsomes due to their different densities. Rough microsomes are denser and can be recovered as a lower band in the sucrose gradient compared to the smooth microsomes. Endogenous RNA attached to the microsomes can be detached from the microsomes, by, e.g., incubating the microsomes with an RNase. Alternatively, the RNA can be removed from the microsomes by incubation of the

microsomes in a high salt solution. In another embodiment, the microsomes are prepared by reconstitution.

Microsomes can be added to a translation system or a solution containing RNA that is at least partially translated at a concentration of from about 1.2 to about 5 A280 U/ml. Microsomes are preferably added at a concentration of about 3, 4, or 5 A280 U/ml.

5 In a specific embodiment, the invention provides for isolation and cloning of an RNA molecule isolated from microsomes that are isolated from a specific cell or tissue sample. For example, microsomes are prepared from a specific cell or tissue sample, e.g., by homogenization of the cell or tissue, low speed centrifugation and centrifugation of the supernatant containing the microsomes in a sucrose density gradient, as further described herein. The rough microsomes are
10 then isolated from the gradient and one or more RNA molecules are isolated therefrom.

Exemplary purification of microsome/RNA complexes and isolation of RNA from the microsome/RNA complexes

15 Following incubation of RNA, an *in vitro* translation system, and microsomes for an appropriate amount of time to generate at least partially translated RNA molecules associated with microsomes, the microsomes are isolated from the reaction mixture.

In one embodiment, the microsomes are isolated from the mixture containing the microsomes, the RNA, and the translation reaction by subjecting the reaction mixture to a sucrose density gradient centrifugation, such that the microsomes and the RNA associated therewith
20 ("microsomes/RNA complexes) form a band, whereas the free ribosomes and other elements of the reaction mixture are pelleted. The band containing the microsomes/RNA complexes, which also contain the polypeptide attached thereto, is isolated from the centrifugation tube. Thus, this fraction will contain the RNA associated with microsomes, i.e., RNA containing a signal sequence.

The RNA associated with the microsomes can be isolated by various methods. In a
25 preferred embodiment, the isolated (bound) polysomes are disrupted by removal of Mg^{2+} , e.g., by dilution, via chelating agents or through the destruction of proteins by any number of methods, e.g., proteases, chlorophorm and/or phenol or any other agent for isolating RNA.

Alternatively, the ribosomes can be first separated from the endoplasmic membrane, e.g.,
by the addition of a detergent. Ribosomes can also be released from the membranes by methods
30 which do not use detergents, as described, e.g., in Harrison et al. (1974) Eur. J. Biochem. 47:613. The RNA can then be separated from the ribosomes, e.g., by using at least one protease or by performing a phenol extraction.

The preparation of RNA encoding proteins having a signal peptide can be enriched in RNA encoding proteins having specific characteristics. Accordingly, the polyribosomes, which contain

RNA and the growing peptidic chain, are separated from the microsomal membranes and screened for polypeptides having a specific activity prior to isolation of the RNA. In this embodiment, it is preferred that translation be continued for a longer time, such that longer peptidic chains are synthesized. It is also preferable in this embodiment to use an agent that cause the polyribosome/RNA/polypeptide to separate from the ER membrane without significantly affecting the polypeptide or the RNA molecules of the complex. The ER membrane can also be dissolved. For example, mild detergents, e.g., non ionic detergents can be used. In an illustrative embodiment, the polyribosomes isolated from microsomes, can be screened for their capacity to interact with a specific molecule, e.g., a receptor. This can be achieved, e.g., by contacting the polyribosomes with a soluble or membrane bound receptor and isolating the polyribosomes which interact with the receptor. The RNA from these polyribosomes, which encode the polypeptide having the specific activity screened for, can then be extracted.

The nascent polypeptidic chain attached to the polyribosome can also be screened for interaction with surface antigens, toxins, organic polymers, antibodies, metabolites, hormones, and active sites of protein molecules. As set forth above, a compound can be linked to a solid surface. A solid surface can be, e.g., a membrane, beads, e.g., magnetic beads, a column or the surface of a plate. The coupling chemistries to link a compound to a specific surface will depend on the compound and the specific surface. For example, many proteins and nucleic acids can be attached to membranes and e.g., cross-linked to the membrane. In preferred embodiments, bovine serum albumin (BSA), gelatin, casein, nonfat milk or other proteinous material is typically added in excess to bind up any "free" surface sites prior to contacting with the polyribosomes. This preabsorption step is likely to lead to much greater binding specificity of the polysomes to the compound of interest. The binding of polysomes to substances of interest may be performed in the presence of $MgCl_2$ (preferably 5 mM) and/or RNase inhibitors. In addition, specific incubation parameters, such as low or high temperature, high or low salt, or different pHs, may be used to identify peptides which bind conditionally, i.e., depending on their environment. Incubation times may depend on the concentration of the bound compound of interest and on the nature of the compound of interest.

After allowing the polysomes to bind to the substance of interest, non-binding polysomes can be removed, e.g., by washings. The wash preferably contains $MgCl_2$ and optionally gelatin, BSA, or other proteinous material to reduce non-specific binding. The RNA from the selected polysomes can then be extracted as described above and used, e.g., for preparing a library of nucleic acids.

In some embodiment, binding of polyribosomes to whole cells or cell extracts is also considered. The polyribosomes can also be screened for a specific biological activity, e.g., an enzymatic activity. In this embodiment, the polyribosomes isolated from the microsomes can be

grouped into pools, which are screened for a specific function, e.g., nuclease or protease activity. Once a positive pool has been identified, the RNA can be extracted, optionally amplified, e.g., by RT-PCR, resubjected to *in vitro* transcription/translation in the presence of microsomes, and rescreened for the particular biological activity of interest. Biological activities of interest also include stimulation or inhibition of cell proliferation, differentiation, and induction of cell death.

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Cloning of RNA molecules isolated from microsomes and/or preparation of a library

In one embodiment of the invention, at least one single stranded DNA molecule is synthesized from at least one RNA molecule isolated from the microsomes.

10 A first strand DNA can be synthesized from RNA by various methods known in the art. In a preferred embodiment of the invention, a first cDNA strand is synthesized by reverse transcription using a reverse transcriptase and a primer, e.g., an oligodT primer.

15 In a preferred embodiment of the invention, the single stranded DNA molecule is converted into a double stranded cDNA molecule. A second cDNA strand can be synthesized according to various methods known in the art. In a preferred embodiment, the second cDNA strand is synthesized by Okayama and Berg method modified by Gubler and Hoffman, in which the product of the first strand synthesis, a cDNA-mRNA hybrid, is used as a template for a nick-translation reaction using RNAase H and a polymerase. According to the method, RNAase H produces nicks and gaps in the mRNA strand of the hybrid, creating a series of RNA primers that are used by *E. coli* DNA polymerase I during the synthesis of the second strand of cDNA.

20 In another embodiment, the second strand is synthesized by self-priming. Accordingly, the cDNA-mRNA hybrid is denatured by boiling or the RNA is hydrolyzed by NaOH and the second strand is primed by the 3' end of the first cDNA strand which forms a hairpin loop. The second strand is then extended from the self-primer by using, e.g., Klenow fragment of *E. coli* DNA Polymerase I. The hairpin loop can then be eliminated by, e.g., digestion with nuclease S1, which
25 degrades single stranded DNA.

In yet another method of the invention, the second strand is synthesized as follows. After completion of the first strand synthesis, terminal transferase is used to add homopolymeric tails of C residues to the free 3' OH end. This tail is then hybridized to oligodG, which serves as a primer for synthesis of a full-length second strand of cDNA.

30 All of the above methods and others for synthesizing a first and second cDNA strand are described in detail, e.g., in Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989).

In a preferred method of the invention, the single or double stranded cDNA is inserted into a vector or a plasmid. In an even more preferred embodiment, a population of cDNAs is cloned in a vector to form a library, e.g., a cDNA library.

5 The first and second strand of cDNA molecules and preparation of a cDNA library can also be constructed using commercially available kits, e.g., the Superscript Plasmid System for cDNA synthesis and Plasmid Cloning Kit (Life Technologies, Gaithersburg, MD).

Yet other methods for cloning the isolated RNA molecule(s) include techniques based on polymerase chain reaction (PCR). For example, the RNA molecules can be reverse transcribed into a first strand DNA, a second strand is synthesized and the DNA is then used in a PCR reaction. Oligonucleotides can be linked to both ends of the DNA prior to amplification with primers
10 hybridizing to these oligonucleotides. One can also use the amplified DNA for further enrichment in DNA sequences encoding proteins containing a signal peptide. For example, the DNA population can be cloned into a vector, the DNA is transcribed into RNA, *in vitro* translated and contacted with microsomes and the RNA associated with the microsomes is isolated.

In one embodiment, the nucleotide sequence of individual clones of the library is
15 determined, to identify secreted proteins. The nucleotide sequence of the isolated nucleic acid and the protein encoded thereby can be compared to the nucleotide and amino acid sequences present in GenBank to determine if the nucleic acid and/or protein is homologous to known nucleic acids and proteins. *In vitro* assays can then be used to determine whether the protein cloned is a secreted protein or a protein associated with a cellular membrane. For example, a nucleic acid
20 encoding a "TAG", e.g., myc tag or a tag consisting of a 16 amino acid portion of the *Haemophilus influenza* hemagglutinin protein (Cross et al. (1984) Mol. Cell. Biol. 4:1834), can be linked to the nucleic acid of interest, the nucleic acid and the tag can be inserted into an expression vector, transfected into host cells and the location of the "TAG" is then determined using a reagent that specifically detects the "TAG". Other useful epitope tags include myc-epitopes (e.g., see Ellison
25 et al (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ) The reagent can be an antibody, e.g., Ab 12CA5, which specifically interacts with the *H. influenza* TAG peptide and can be obtained, e.g., from Babco, Richmond CA. The location of the tagged protein can be determined, e.g., by monitoring the presence of the TAG in the
30 supernatant, such as by immunoprecipitations using the supernatant of the transfected cells. The presence of the protein in the supernatant of the cell culture will be indicative that the protein is in fact a secreted protein. If the protein is not a secreted protein, the cellular location of the protein can be determined by performing immunohistochemistry experiments using a reagent binding specifically to the TAG, e.g., an antibody. These assays will indicate whether the cloned nucleic

acid encodes a secreted protein or a membrane associated protein, as well as to which membrane the protein is associated.

Various assays known in the art can be used to determine the activity of the newly cloned gene. Some available assays are described, e.g., in U.S. 5,728,819 by Jacobs et al. issued on March 17, 1998.

5 In another embodiment, the cDNA library is screened with a specific probe. For example, if one wishes to isolate a nucleic acid encoding a secreted protein that is homologous to a known secreted protein, the cDNA library can be screened under low stringency conditions with a nucleic acid encoding the protein.

10 In another preferred embodiment of the invention, the cDNA library is an expression library. The expression library can be a prokaryotic expression library, e.g., a bacterial expression library. Alternatively, the expression library can be a eukaryotic expression library, which can be introduced and expressed, i.e., in yeast cells, or in vertebrate cells, such as mammalian cells, preferably human cells. An expression library can be screened by various methods, e.g., binding of the secreted protein to a ligand, receptor, or antibody, or a biological function, as set forth below.

15 In a preferred embodiment, the expression library is divided into pools of clones, which are screened to identify a particular pool, which is then divided into subpools, which are screened, redivided several times until one or more clones having the desired activity are isolated. The screening method can be based on a specific biological function, e.g., screening with a receptor for the identification of a ligand of a receptor, e.g., an orphan receptor. The screening method can also be based on a specific biological activity, e.g., an enzymatic activity, e.g., stimulation or inhibition of cellular growth, stimulation or inhibition of differentiation of one or more cell types, induction or prevention of apoptosis, degradation of the extracellular matrix, and others.

20 Expression vectors of utility in the present invention are often in the form of "plasmids", which refer to circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

25 Expression vectors useful in the present invention typically contain an origin of replication, a promoter located in front of (i.e., upstream of) the DNA sequence and followed by the DNA sequence of interest, transcription termination sequences and the remaining vector. The expression vectors may also include other DNA sequences known in the art, for example, stability leader sequences which provide for stability of the expression product, secretory leader sequences which provide for secretion of the expression product, sequences which allow expression of the structural gene to be modulated (e.g., by the presence or absence of nutrients or other inducers in the growth medium), marking sequences which are capable of providing phenotypic selection in transformed

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host cells, stability elements such as centromeres which provide mitotic stability to the plasmid, and sequences which provide sites for cleavage by restriction endonucleases. The characteristics of the actual expression vector used must be compatible with the host cell which is to be employed. For example, when cloning in a mammalian cell system, the expression vector should contain promoters isolated from the genome of mammalian cells, (e.g., mouse metallothionien promoter), or from viruses that grow in these cells (e.g., vaccinia virus 7.5K promoter). An expression vector as contemplated by the present invention is at least capable of directing the replication, and preferably the expression, of the DNA sequences of the present invention. Suitable origins of replication include, for example, a yeast ARS element, a COLEI ori element, and an SV40 ori element. Suitable promoters include, for example, the yeast GAL1 promoter, the synthetic bacterial Tac promoter, the SV40 early promoter and the baculovirus AcNPV polyhedrin promoter. Suitable termination sequences include, for example, the yeast cycl terminator, the bacterial rrnB terminator, and the SV40 3' polyA' signal segment. As selectable markers, kanamycin resistance, uracil (URA3) marker and neomycin resistance can be employed. All of these materials are known in the art and are commercially available.

Preferred expression vectors contain a "TAG" sequence located either upstream or downstream of the restriction sites for inserting the DNA of interest, such that the DNA of interest is expressed as a TAG fusion protein. The presence of a TAG on a protein facilitates detection and purification of the protein. In one embodiment, the TAG is a myc tag. In another embodiment, the TAG is a hexahistidine tag, which binds with high affinity to nickel.

Suitable expression vectors containing the desired coding and control sequences may be constructed using standard recombinant DNA techniques known in the art, many of which are described in Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989).

In another embodiment, the RNA isolated from the microsomes is amplified and subjected to a second round of screening for signal sequences. For example, the RNA molecules can be amplified using RNA-directed RNA polymerases. Alternatively, cDNA can be prepared from the RNA, the cDNA cloned into a vector downstream of a promoter, e.g., SP6, T3, or T7 promoter, such that the cloned cDNA can be *in vitro* transcribed. The *in vitro* transcribed RNA, which has preferably been capped during the *in vitro* transcription reaction, can then be *in vitro* translated and contacted with microsomes as described in the first round of selection. Yet further rounds of enrichment can be performed.

Accordingly, the method of the invention permits isolation of nucleic acids encoding proteins having a signal sequence, e.g., secreted proteins. Secreted proteins can be cell growth and/or differentiation factors, e.g., cytokines, interleukins, colony stimulating factors and others.

The invention further provides cells, also termed herein "host cells" comprising a vector including a cloned nucleic acid of the invention. Such cells can be used, e.g., for the production of high amounts of secreted proteins, which can be used, e.g., for preparing pharmaceutical compositions. Host cells can be prokaryotic, e.g., bacterial, or eukaryotic, e.g., yeast cells, e.g., *S. cerevisiae*, and *S. pombe*, vertebrate cells, preferably mammalian cells, even more preferably human cells. Expression in a eukaryotic host cells has the advantage that proteins can be posttranslationally modified, e.g., glycosylated. Preferred eukaryotic cells for expression of a protein of the invention include COS monkey cells and CHO cells.

The invention further provides proteins encoded by the nucleic acids of the invention. Such proteins can be produced, e.g., from host cells transfected with the nucleic acids of the invention. Proteins can be produced according to methods known in the art, such as by culturing host cells comprising a nucleic acid of the invention in conditions suitable for the production of the protein encoded by the nucleic acid, and isolation of the protein from the culture medium and/or the host cells.

Also within the scope of the inventions are non-human transgenic animals containing a nucleic acid identified according to the method of the invention. The term transgenic animals includes animals in which a nucleic acid of the invention is introduced into the animal and is integrated into the genome of the animal or an animal in which the endogenous gene corresponding to a gene of the invention has been mutated, e.g., by the insertion of an unrelated nucleic acid (e.g., "knock-out" mutant).

The invention also provides kits containing reagents for performing the invention. Such reagents can include one or more of: an *in vitro* transcription system, an *in vitro* translation system, microsomes, and reagents and buffers for isolating the microsome/polyribosome complexes and/or the RNA, reagents for synthesizing a first stand DNA, reagents for synthesizing a second strand of DNA, reagents for preparing a library, and oligonucleotides and primers for amplification of the DNA. Other kits within the scope of the invention contain one or more of the newly cloned DNA molecules.

Exemplary uses of the invention

The nucleic acids of the invention, e.g., encoding secreted proteins, can have various utilities, in particular therapeutic utilities. Sequence analysis of the nucleic acid and amino acid sequences will reveal whether the nucleic acid and/or amino acid sequence have any particular motifs characteristic of certain types of proteins. Furthermore, comparison of the nucleic acid and amino acid sequences with known sequences will reveal whether the newly cloned nucleic acid or protein has any homology to known nucleic acids or proteins. In addition, *in vitro* and *in vivo*

experiments can be performed to determine whether the nucleic acid encodes a protein having a particular biological activity, e.g., cell growth and/or differentiation stimulatory activity. Thus, based on at least this information, the nucleic acid and/or protein encoded thereby can be used for treating or preventing specific diseases or disorders. For example, if the protein has a cell growth stimulatory activity, the protein could be used for treating cell proliferative disorders, e.g., cancer.

5 The polynucleotides provided by the present invention can be used by the research community for various purposes. The polynucleotides can be used to express recombinant protein for analysis, characterization or therapeutic use; as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in disease states); as molecular weight markers on Southern gels;
10 as chromosome markers or tags (when labeled) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in patients to identify potential genetic disorders; as probes to hybridize and thus discover novel, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; as a probe to "subtract-out" known sequences in the process of discovering other novel polynucleotides; for selecting and making
15 oligomers for attachment to a "gene chip" or other support, including for examination of expression patterns; to raise anti-protein antibodies using DNA immunization techniques; and as an antigen to raise anti-DNA antibodies or elicit another immune response. Where the polynucleotide encodes a protein which binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the polynucleotide can also be used in interaction trap assays (such as,
20 for example, that described in Gyuris et al., Cell 75:791-803 (1993)) to identify polynucleotides encoding the other protein with which binding occurs or to identify inhibitors of the binding interaction.

 The proteins provided by the present invention can similarly be used in assay to determine biological activity, including in a panel of multiple proteins for high-throughput screening; to raise
25 antibodies or to elicit another immune response; as a reagent (including the labeled reagent) in assays designed to quantitatively determine levels of the protein (or its receptor) in biological fluids; as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in a disease state); and, of course, to isolate correlative receptors or ligands. Where the protein binds or potentially
30 binds to another protein (such as, for example, in a receptor-ligand interaction), the protein can be used to identify the other protein with which binding occurs or to identify inhibitors of the binding interaction. Proteins involved in these binding interactions can also be used to screen for peptide or small molecule inhibitors or agonists of the binding interaction.

Methods for performing the uses listed above are well known to those skilled in the art. References disclosing such methods include without limitation "Molecular Cloning: A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory Press, Sambrook, J., E. F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning Techniques", Academic Press, Berger, S.L. and A. R. Kimmel eds., 1987.

5 Any or all of these research utilities are capable of being developed into reagent grade or kit format for commercialization as research products. In one embodiment, the invention provide a solid surface, e.g., a chip, to which are linked one or more polynucleotide or polypeptide cloned according to the method of the invention. Such solid surfaces can be commercialized and used by others for characterizing other proteins. In particular, these should be useful for identifying ligands
10 of orphan receptors.

Polynucleotides and proteins of the present invention can also be used as nutritional sources or supplements. Such uses include without limitation use as a protein or amino acid supplement, use as a carbon source, use as a nitrogen source and use as a source of carbohydrate. In such cases the protein or polynucleotide of the invention can be added to the feed of a particular organism or
15 can be administered as a separate solid or liquid preparation, such as in the form of powder, pills, solutions, suspensions or capsules. In the case of microorganisms, the protein or polynucleotide of the invention can be added to the medium in or on which the microorganism is cultured.

In one embodiment of the invention, a disease or disorder is prevented or treated in a subject, by administering to the subject an effective amount of a pharmaceutical composition
20 comprising a secreted protein or a nucleic acid encoding a secreted protein and a pharmaceutically acceptable carrier. The nucleic acid can be a nucleic acid encoding the full length protein or a biologically active portion thereof. In one embodiment, the nucleic acid is operably linked to a promoter, such that upon entrance into a cell, the nucleic acid is transcribed and translated into a biologically effective protein. The promoter can be a constitutive promoter or an inducible
25 promoter. In another embodiment, the protein is synthesized *ex vivo* and administered in an effective amount to the subject having a disease or disorder. The protein can be synthesized in any cell, e.g., prokaryotic cell, such as a bacterial cell, or a eukaryotic cell, e.g., a human cell according to methods known in the art.

Nucleic acids or proteins of the invention can be administered to a subject at therapeutically
30 effective dose to treat or ameliorate a specific disease. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms associated with the particular disease.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or

excipients. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by, for example, injection, inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For such therapy, the nucleic acids and proteins of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the nucleic acids and proteins of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the nucleic acids and proteins of the invention may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the nucleic acids and proteins of the invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be

formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

5 The nucleic acids and proteins of the invention may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

10 The nucleic acids and proteins of the invention may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

15 In addition to the formulations described previously, the nucleic acids and proteins of the invention may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

20 Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the nucleic acids and proteins of the invention are formulated into ointments, salves, gels, or creams as generally known in the art.

25 In clinical settings, the gene delivery systems for the therapeutic gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) PNAS 91: 3054-3057). A therapeutic gene, can also be delivered

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in a gene therapy construct by electroporation using techniques described, for example, by Dev et al. ((1994) Cancer Treat Rev 20:105-115).

5 The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can
comprise one or more cells which produce the gene delivery system.

10 The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

15 In another embodiment, the secreted proteins isolated according to the method of the invention can be distributed in 96 well plates and made commercially available for high throughput screening of the proteins for a specific activity. Accordingly, in a specific embodiment, the secreted proteins are produced *in vitro*, e.g., in bacteria, and are then linked to the surface of the 96 well plate and made commercially available. Thus, high numbers of secreted proteins can be screened,
e.g., for specific binding to a receptor.

20 The contents of all cited references (including literature references, issued patents, published patent applications as cited throughout this application are hereby expressly incorporated by reference. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology,
microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985);
25 Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.);
30 Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and

C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Examples

5 **Example 1: Separation of RNA encoding secreted proteins from RNA encoding non-secreted proteins**

10 This example demonstrates that RNA encoding a secreted protein can be separated from RNA encoding a non-secreted protein. The technique used, which is outlined in Figure 1, is based on the fact that secreted proteins are bound to microsomes as they are being translated, bringing along their associated RNAs. The membrane vesicles (and associated RNAs) can then be isolated on a discontinuous sucrose density gradient which causes the vesicles to float towards the top of the gradient, whereas the ribosomes, free polysomes, and other RNAs remain at the bottom of the gradient due to their higher densities. RNA molecules associated with microsomes can then be isolated.

15 Capped RNA encoding the secreted protein FGF Related Ligand 2 (FRL2; Noriyuki et al. *Cell* 83:621) and RNA encoding the cytosolic protein ornithine decarboxylase (ODC; Bassez et al. *Development* 110:955) were each prepared separately as follows. Plasmid (pCS2+; Turner and Weintraub (1994) *Genes Dev.* 8:1434) containing DNA encoding each of the proteins were linearized downstream of the insert with the restriction enzyme NotI and used as template for the *in vitro* synthesis of RNA as described in Krieg and Melton (1984) *Nucl. Acid. Res.* 12, 7057-7070.

20 To show that the RNAs encoding the two proteins can be separated from each other, a mixture of the two RNAs was subjected to the procedure outlined in Figure 1. Accordingly, 0.25 µg of each of the synthesized RNAs were then mixed together and the mixture was incubated for 1 hour at 30°C in a 30 µl reaction containing 20 µl nuclease treated rabbit reticulocyte lysate (Promega), 45 units of Rnasin (Promega), 15 µM complete amino acid mix (Promega), and 0.2 mg/ml rabbit liver tRNA (Sigma) with or without 2 µl canine pancreatic microsomal membranes (Promega). After the incubation, the reactions were mixed with 90 µl of dilution buffer (50 mM Hepes, pH 7.5; 500 mM KCl; 5 mM MgCl₂; 1 mM DTT; 1 mg/ml rabbit liver tRNA) containing 2.9 M sucrose (final sucrose concentration of the mixtures was about 2.2M). The mixtures were then applied to the bottom of Beckman Ultra-Clear™ 5 X 41 mm centrifuge tubes and layered with 150 µl, 200 µl, 50 µl, and 25 µl of dilution buffer containing, 2.0 M, 1.8 M, 0.3 M, and 0.0 M Sucrose respectively. Samples were then centrifuged in a Beckman Sw50 ultracentrifuge rotor at 50,000 rpm for 5 hours at 4°C. After centrifugation, three fractions (180 µl each) were collected

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successfully by pipetting from the top of the centrifuge tube and processed for reverse transcription-polymerase chain reaction (RT-PCR).

Each of the fractions were extracted with 1 mL of Rnazol B (Tel-Test) followed by extraction with an equal volume of chloroform. Total RNAs were precipitated with 2-propanol, washed with 70% ethanol, and resuspended with 14 μ l of water containing 0.05 units of a reverse primer encoding a common vector sequence (5' ACC TCC CCC TGA ACC TGA AAC 3'; SEQ ID NO: 1) present within the 3' untranslated region of all *in vitro* transcripts generated from the pCS2+ vector. Reverse transcription was performed with the addition of RT buffer (1X final; Promega), 1 mg/ml BSA, 1 mM DTT, 25 mM dNTP, 20 units RNasin, and 100 units MMLV-RT (Promega). Samples were incubated at 42°C for 30 minutes. PCR was carried out in 10 μ l reactions consisting of 0.5 units Taq DNA polymerase (Promega), 2.5 mM MgCl₂, 100 μ M dNTP, 0.5 μ Ci [α -³²-P] dCTP, 1 μ l of the reverse transcribed reaction, and 10 pmol of each primer. The following primers were used in the PCR reactions: FRL2 forward primer 5' ATG CTT GAC ATT ATG GTG 3' (SEQ ID NO: 2); FRL2 reverse primer 5' TTA TCT CAT AGC AGG GAG 3' (SEQ ID NO: 3); ODC forward primer 5' AAT GGA TTT CAG AGA CCA 3' (SEQ ID NO: 4); and ODS reverse primer 5' CCA AGG CTA AAG TTG CAG 3' (SEQ ID NO: 5). 29 cycles were performed at the following temperatures: 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute. A sample of each of the PCR reactions was then separated on an agarose gel and ethidium bromide stained to visualize the amplified products.

A photograph of the agarose gel is shown in Figure 2. The results indicate that in the absence of microsomes, messages encoding both FRL2 and ODC are present only at the bottom of the gradient. In the presence of microsomes, however, FRL2 (encoding a secreted protein) is found both at the top and bottom of the gradient, whereas ODC (encoding a cytosolic protein) is found exclusively at the bottom of the gradient.

Thus, this Example demonstrates that the procedure outlined in Figure 1 allows the isolation of RNA encoding secreted proteins from RNA encoding non-secreted proteins.

Example 2: Isolation of RNA encoding secreted proteins from libraries

This example demonstrates that the technique used in the previous example and outlined in Figure 1 can be used to isolate RNAs encoding secreted proteins or other proteins having a signal peptide from a mixture of RNAs transcribed from a cDNA library.

Capped RNA was prepared from a from a *Xenopus* stage 11-15 library as follows. The library was built into the vector pCS2+ (Turner and Weintraub (1994) *Genes Dev.* 8, 1434-1447) and was linearized with the restriction enzyme Not I to synthesize capped RNA *in vitro* as described previously (Krieg and Melton (1984), *supra*). For floating, 0.5 μ g sample of the

synthesized RNA was incubated for 1 hour at 30°C in 30µl reactions containing 20µl nuclease treated rabbit reticulocyte lysate (Promega), 45 units of Rnasin (Promega), 15µM complete amino acid mix (Promega), 2µl canine pancreatic microsomal membranes (Promega), and 0.2 mg/ml rabbit liver tRNA (Sigma). The final reaction was then mixed with 90µl of dilution buffer (50 mM Hepes, pH 7.5; 500 mM KCl; 5 mM MgCl₂; 1 mM DTT; 1mg/ml rabbit liver tRNA) containing 2.9 M sucrose (final sucrose concentration was about 2.2M). The mixture was then applied to the bottom of Beckman Ultra-Clear™ 5 X 41 mm centrifuge tubes and layered with 150µl, 200µl, 50µl, and 25µl of dilution buffer containing, 2.0 M, 1.8 M, 0.3 M, and 0.0 M Sucrose respectively. Samples were then centrifuged in a Beckman Sw50 ultracentrifuge rotor at 50,000 rpm for 5 hours at 4°C. After centrifugation, 5 fractions (110µl each) were collected successively by pipetting from the top of the centrifuge tubes and processed for RT-PCR.

After centrifugation, each of the fractions were extracted with 1 mL of Rnazol B (Tel-Test) followed by extraction with an equal volume of chloroform. Total RNAs were precipitated with 2-propanol, washed with 70% ethanol, and resuspended with 14µl of water containing 0.05 units of a reverse primer encoding a common vector sequence present within the 3' untranslated region of all transcripts (5' ACC TCC CCC TGA ACC TGA AAC 3'; SEQ ID NO: 1). Reverse transcription was performed with the addition of RT buffer (1X final; Promega), 1 mg/ml BSA, 1 mM DTT, 25 mM dNTP, 20 units RNasin, and 100 units MMLV-RT (Promega). Samples were incubated at 42°C for 30 minutes. PCR was carried out in 10µl reactions consisting of 0.5 units Taq DNA polymerase (Promega), 2.5 mM MgCl₂, 100µM dNTP, 0.5 µCi [³²-P] dCTP, 1µl of the reverse transcribed reaction, and 10 pmol of each primer. The sequences encoding the following secreted or cytosolic proteins were amplified: the secreted proteins Sonic Hedgehog, Epidermal Fibroblast Growth Factor (EFGF), Sizzled, Dickkopf, Follistatin, and Cerberus; and the cytosolic proteins: epidermal keratin (epid. Keratin), EF1alpha, Xtwist, Goosecoid, and Neurogenin. The following primers were used in the PCR reactions for detecting RNA encoding each of these proteins: *sonic hedgehog* forward 5'-TCCTCCAGACCAGACCTTCCA-3' (SEQ ID NO: 6), reverse 5'-CGTATTAGTTCGTTTGTGTG-3' (SEQ ID NO: 7); *eFGF* forward 5'-GTGCCTGCCGCTTTCTTTCCA-3' (SEQ ID NO: 8), reverse 5'-AACTCAGACTCCAACCTTCCA-3' (SEQ ID NO: 9); *sizzled* forward 5'-AGCCCTGCCGTAGTATGTGTG-3' (SEQ ID NO: 10), reverse 5'-CTGTGGGTCTGGTCCGTATCA-3' (SEQ ID NO: 11); *dickkopf* forward 5'-AGATGATGACTGTGCCCTTGA-3' (SEQ ID NO: 12), reverse 5'-CCTTTCCTCTGTGCTTGGTG-3' (SEQ ID NO: 13); *follistatin* forward 5'-CAGTGCAGCGCTGGAAAG-3' (SEQ ID NO: 14), reverse 5'-TGCGTTGCGGTAATTCAC-3' (SEQ ID NO: 15); *cerberus* forward 5'-TTCTCGCCGTTCTTTTGATAA-3' (SEQ ID NO: 16),

reverse 5'-ACACGTGCATTCCTCTACCAT-3' (SEQ ID NO: 17). *epidermal keratin* forward 5'-CACCAGAACACAGATAC-3' (SEQ ID NO: 18), reverse 5'-CAACCTTCCCATCAACCA-3' (SEQ ID NO: 19); *EFlalpha* forward 5'-CAGATTGGTGCTGGATATGC-3' (SEQ ID NO: 20), reverse 5'-ACTGCCTTGATGACTCCTAG-3' (SEQ ID NO: 21); *Xtwist* forward 5'-AGTCCGATCTCAGTGAAGGGCA-3' (SEQ ID NO: 22), reverse 5'-TGTGTGTGGCCTGAGCTGTAG -3' (SEQ ID NO: 23); *goosecoid* forward 5'-ACAACTGGAAGCACTGGA-3' (SEQ ID NO: 24), reverse 5'-TCTTATTCCAGAGGAACC-3' (SEQ ID NO: 25); and *neurogenin* forward 5'-CAAGAGCGGAGAACTGTGT-3' (SEQ ID NO: 26), reverse 5'-GAAGGAGCAACAAGAGGAAG-3' (SEQ ID NO: 27). 29 cycles were performed at the following temperatures: 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute. A sample of each reaction was then subjected to agarose gel electrophoresis.

Figures 3 and 4 shows PCR amplification products obtained using primers detecting secreted (Figure 3) and cytosolic (Figure 4) proteins, from RNA samples obtained from the top and from the bottom of the sucrose gradient. The results show that all the messages encoding secreted proteins that were tested were detected at the top of the gradient. The fact that the degree of floating varies somewhat for each message, probably reflects the efficiency of the translation machinery of the rabbit reticulocyte lysate, the processing of the translating proteins by the microsomal membranes, or both. Differences in the intensity of the signal between the different messages probably reflects the relative abundance of the messages in the library. Figure 4 shows that, for messages encoding the non-secreted proteins that were tested, all were detected at the bottom of the gradient, but not at the top. Thus, RNAs encoding proteins which do not contain a signal peptide are never present at the top of the sucrose gradient. These results indicate that the method developed is able to select for RNAs encoding secreted proteins and that, more importantly, this method is able to select against those messages encoding cytoplasmic proteins. The results also show that this method can be generally applied to preexisting libraries, as shown for the staged *Xenopus* library.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein.

Such equivalents are intended to be encompassed by the following claims.

Claims:

1. A method for isolating a nucleic acid encoding a protein having a signal peptide, comprising isolating an RNA molecule associated with microsomes under conditions wherein the RNA is at least partially translated.
- 5 2. The method of claim 1, wherein the protein having a signal peptide is a secreted protein.
3. The method of claim 1, further comprising synthesizing a single stranded DNA molecule from the RNA molecule.
- 10 4. The method of claim 3, further comprising converting the single stranded DNA molecule into a double stranded DNA molecule.
- 15 5. The method of claims 1, 3, or 4, further comprising inserting the RNA, the single stranded DNA molecule or the double stranded DNA molecule into a vector.
6. The method of claim 5, wherein the vector is an expression vector.
7. The method of claim 6, further comprising producing a protein from the expression vector.
- 20 8. The method of claim 7, further comprising preparing a pharmaceutical composition comprising the protein and a pharmaceutically acceptable carrier.
9. The method of claim 1, wherein the microsomes and the RNA are from the same cell.
- 25 10. The method of claim 9, wherein the cell is a mammalian cell.
11. The method of claim 10, wherein the mammalian cell is a human cell.
- 30 12. The method of claim 1, 3, or 4, wherein the microsomes and the RNA are heterologous.
13. The method of claim 1 or 12, wherein the RNA which is at least partially translated is an RNA which is translated in an *in vitro* translation system.

14. The method of claim 13, wherein the *in vitro* translation system is a reticulocyte lysate.
15. The method of claim 12, wherein the RNA which is at least partially translated is RNA isolated from a cell or tissue sample.
- 5 16. The method of claim 15, wherein the cell or tissue is a mammalian cell or tissue.
17. The method of claim 16, wherein the mammalian cell or tissue is a human cell or tissue.
- 10 18. The method of claim 12, wherein the RNA, the *in vitro* translation system and the microsomes are contacted, to thereby produce RNA which is partially translated and wherein RNA encoding a protein comprising a signal peptide is associated with the microsomes.
- 15 19. The method of claim 1 or 18, further comprising isolating the microsomes and the RNA associated therewith.
- 20 20. The method of claim 19, wherein the microsomes are isolated by sucrose density gradient centrifugation.
- 20 21. A method for isolating a nucleic acid encoding a protein having a signal peptide, comprising:
- (i) contacting one or more RNA molecules with an *in vitro* translation system, to thereby produce one or more RNA molecules which are at least partially translated;
- 25 (ii) contacting the one or more RNA molecules which are at least partially translated with microsomes, such that the RNA molecules which are at least partially translated and which comprise a signal sequence associate with the microsomes, to thereby produce at least one RNA molecule associated with the microsomes;
- (iii) isolating the microsomes and the at least one RNA molecule associated with the microsomes; and
- 30 (iv) isolating at least one RNA molecule associated with the microsomes.
22. The method of claim 21, further comprising synthesizing a single stranded DNA molecule from the RNA molecule.

23. The method of claim 22, further comprising converting the single stranded DNA molecule into a double stranded DNA molecule.
24. The method of claim 23, further comprising inserting the double stranded DNA molecule into a vector.
- 5 25. The method of claim 24, wherein the vector is an expression vector.
26. The method of claim 25, further comprising producing a protein from the expression vector.
- 10 27. The method of claim 26, further comprising preparing a pharmaceutical composition comprising the protein and a pharmaceutically acceptable carrier.
28. A method for preparing a library of nucleic acids encoding proteins comprising a signal peptide, comprising isolating RNA molecules associated with microsomes under conditions wherein the RNA molecules are at least partially translated.
- 15 29. A method for enriching a population of RNA molecules in RNA molecules encoding a protein comprising a signal peptide, comprising
- (i) contacting the population of RNA molecules with a translation system, to
- 20 thereby produce at least partially translated RNA molecules;
- (ii) contacting the at least partially translated RNA molecules with microsomes under conditions in which the partially translated RNA molecules comprising a signal peptide associate with the microsomes;
- (iii) isolating the microsomes and the RNA molecules associated with the
- 25 microsomes; and
- (iv) isolating the RNA molecules from the microsomes, to thereby produce a population of RNA molecules enriched in RNA molecules encoding a protein comprising a signal peptide.
- 30 30. A library of nucleic acids encoding proteins comprising a signal peptide, prepared according to the method of claim 5.
31. The library of claim 30, which is a cDNA library.

32. The library of claim 31, which is an expression library.
33. A library of nucleic acids encoding proteins comprising a signal peptide, prepared according to the method of claim 21.
- 5 34. The library of claim 33, which is a cDNA library.
35. The library of claim 34, which is an expression library.
36. An isolated nucleic acid isolated according to the method of claim 3.
- 10 37. The isolated nucleic acid of claim 36, which encodes a secreted protein.
38. An isolated nucleic acid isolated according to the method of claim 22.
- 15 39. The isolated nucleic acid of claim 38, which encodes a secreted protein.
40. An isolated polypeptide encoded by the isolated nucleic acid of claim 38.
41. An isolated polypeptide encoded by the isolated nucleic acid of claim 38.
- 20 42. A pharmaceutical composition comprising a nucleic acid of claim 36 and a pharmaceutically acceptable carrier.
43. A pharmaceutical composition comprising a nucleic acid of claim 38 and a pharmaceutically acceptable carrier.
- 25 44. A pharmaceutical composition comprising a polypeptide of claim 40 and a pharmaceutically acceptable carrier.
- 30 45. A pharmaceutical composition comprising a polypeptide of claim 41 and a pharmaceutically acceptable carrier.
46. A method for treating a subject for a disease or disorder that can be ameliorated by administration of a secreted protein identified by the method of claim 1, comprising

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administering to the subject an effective amount of a pharmaceutical composition comprising a protein identified by the method of claim 1, such that the disease or disorder is ameliorated in the subject.

- 5 47. A method for treating a subject for a disease or disorder that can be ameliorated by administration of a secreted protein identified by the method of claim 1, comprising administering to the subject an effective amount of a pharmaceutical composition comprising a nucleic acid encoding a protein identified by the method of claim 1, such that the disease or disorder is ameliorated in the subject.

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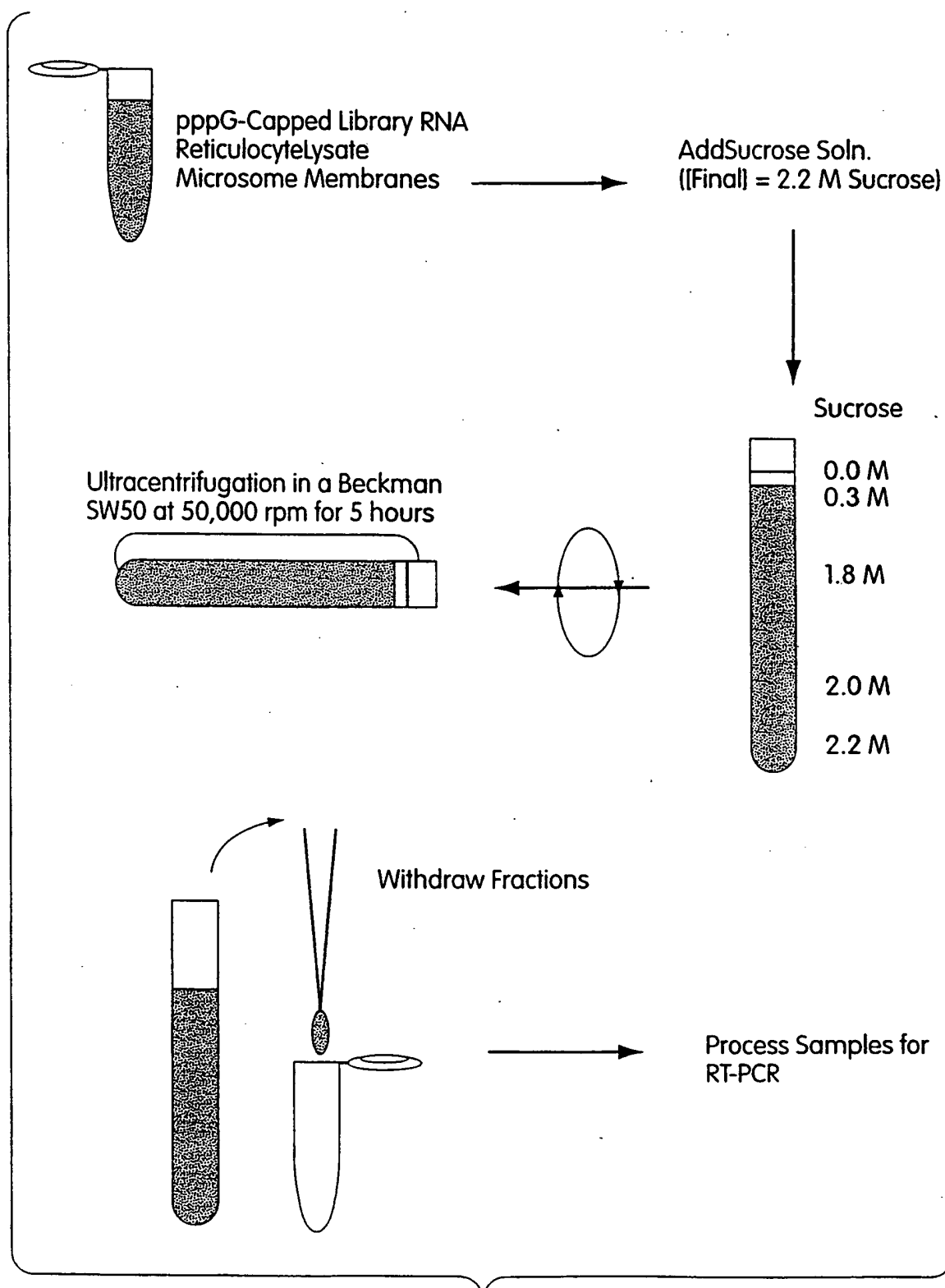
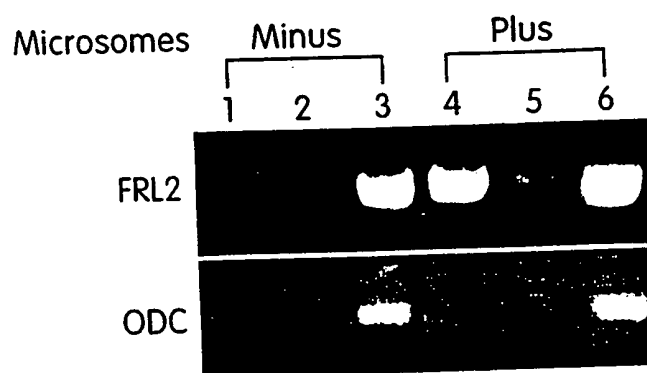


Fig. 1

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Floating is Microsome Dependent



1 and 4 -- Top of the gradient
2 and 5 -- Middle of the gradient
3 and 6 -- Bottom of the gradient

Fig. 2

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RT-PCR of Messages Encoding Secreted Proteins

Sonic Hedgehog



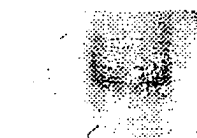
T B

Sizzled



T B

Follistatin



T B

eFGF



T B

Dickkopf



T B

Cerberus



T B

T = Top of sucrose gradient

B = Bottom of sucrose gradient

RNAs were generated from a Xenopus library (Stages 11-15)

Fig. 3

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RT-PCR of Messages Encoding Nonsecreted Proteins

Epid. Keratin



T B

EF1alpha.



T B

Xtwist



T B

Goosecoid



T B

Neurogenin



T B

T = Top of sucrose gradient

B = Bottom of sucrose gradient

RNAs were generated from a Xenopus library (Stages 11-15)

Fig. 4

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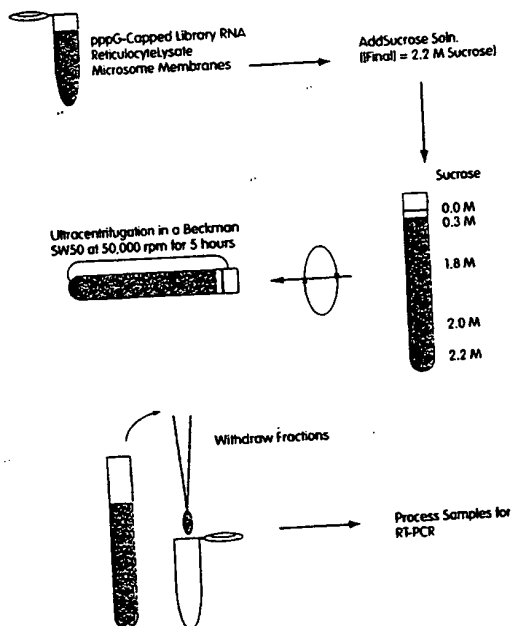
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| | | | |
|---|--|--|--|
| (51) International Patent Classification ⁶ : C12N 15/10, 15/12, 15/85, 5/10, C07K 14/47, A61K 38/17, 48/00 | | A3 | (11) International Publication Number: WO 99/05256 (43) International Publication Date: 4 February 1999 (04.02.99) |
| (21) International Application Number: PCT/US98/15394 (22) International Filing Date: 24 July 1998 (24.07.98) (30) Priority Data: 60/053,586 24 July 1997 (24.07.97) US (71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US). (72) Inventors: KIRSCHNER, Mark, W.; 20 Gralynn Road, Newton, MA 02159 (US). KINOSHITA, Noriyuki; Apartment #202, 3-28-22, Sakae-cho, Fuchu-shi, Tokyo 183 (JP). (74) Agents: CLAUSS, Isabelle, M. et al.; Foley, Hoag & Eliot, LLP, One Post Office Square, Boston, MA 02109-2170 (US). | | (81) Designated States: CA, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report. (88) Date of publication of the international search report: 5 August 1999 (05.08.99) | |

(54) Title: METHOD FOR CLONING SECRETED PROTEINS

(57) Abstract

The invention provides a method for isolating nucleic acids encoding proteins comprising a signal peptide, e.g. a secreted protein. The method of the invention comprises isolating an RNA molecule associated with an endoplasmic reticulum membrane preparation under conditions wherein the RNA is at least partially translated. The invention also provides a library of nucleic acids encoding proteins comprising a signal peptide. Individual nucleic acids isolated according to the method of invention, peptides encoded thereby, pharmaceutical compositions comprising such and kits for performing the method of the invention.



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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/15394

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/10 C12N15/12 C12N15/85 C12N5/10 C07K14/47
A61K38/17 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | US 5 536 637 A (JACOBS KENNETH) 16 July 1996 see the whole document | 1-47 |
| A | WO 96 40904 A (ZYMOGENETICS INC) 19 December 1996 see the whole document | 1-47 |
| -/-- | | |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

21 May 1999

Date of mailing of the international search report

31/05/1999

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

...ernational application No.

PCT/US 98/ 15394

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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Remark: Although claims 46, 47
are directed to a method of treatment of the human/animal
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3. ☐ Claims Nos.:
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Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. l. Application No

PCT/US 98/15394

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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